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Individual and combined *in vitro* (anti)androgenic effects of certain food additives and cosmetic preservatives

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ABSTRACT

The individual and combined (binary mixtures) (anti)androgenic effect of butylparaben (BuPB), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) was evaluated using the MDA-kb2 cell line. Exposing these cells to AR agonists results in the expression of the reporter gene (encoding for luciferase) and luminescence can be measured in order to monitor the activity of the reporter protein. In case of the evaluation of the anti-androgenic effect, the individual test compounds or binary mixtures were tested in the presence of a fixed concentration of a strong AR agonist (1000 pM 5-alpha-dihydrotestosterone; DHT). Cell viability was assessed using a resazurin based assay. For PG, this is the first report in the literature concerning its (anti)androgenic activity. In case of both individual and mixture testing none of the compounds or binary combinations showed androgenic activity. When tested in the presence of DHT, BuPB, BHA and BHT proved to be weak anti-androgens and this was confirmed during the evaluation of binary mixtures (BuPB + BHA, BuPB + BHT and BHA + BHT). Besides performing the *in vitro* testing of the binary combinations, two mathematical models (dose addition and response addition) were evaluated in terms of accuracy of prediction of the anti-androgenic effect of the selected binary mixtures. The dose addition model guaranteed a good correlation between the experimental and predicted data. However, no estimation was possible in case of mixtures containing PG, due to the lack of effect of the compound in case of the individual testing.

1. Introduction

Currently, there is a major concern regarding the role of endocrine disruptors (EDs) in the increased incidence of male reproductive tract disorders (*e.g.* cryptorchidism, hypospadias), decreased sperm production and sperm quality, and testicular cancer (Bergman et al., 2012). Many of these disorders may arise from exposure to disruptors of sex steroid signaling during critical developmental stages. Any compound able to interfere with the biosynthesis, metabolism or action of androgen hormones can affect the differentiation and development of the male reproductive system and may cause testicular dysgenesis syndrome (Bay et al., 2006; Bergman et al., 2012; Gray et al., 2006; Luccio-Camelo and Prins, 2011; Sharpe, 2006; Skakkebaek et al., 2001; Sultan et al., 2001).

There are many chemicals that can act as EDs, including certain food additives and cosmetic preservatives (parabens being the most studied group from cosmetics preservatives) (Błędzka et al., 2014). *In vitro*, these chemicals were able to bind nuclear estrogen (ER) or androgen receptors (AR) and to induce or block the signaling through these receptors (Amadasi et al., 2009; Okubo and Kano, 2003; Schrader and Cooke, 2000; ter Veld et al., 2006). One such compound is propyl gallate (PG) (Fig. 1) (Amadasi et al., 2009; ter Veld et al., 2006), which is used as an antioxidant in food, cosmetics, pharmaceuticals and packaging materials. In food, PG can be used alone or in combination with butylated hydroxyanisole (BHA) and/or butylated hydroxytoluene (BHT) (Fig. 1) (Shahidi and Zhong, 2005) which makes co-exposure very likely. The acceptable daily intake (ADI) for PG is below 0.5 mg/kg/day. However, the exposure estimates indicated values above the ADI in case of adults and the elderly (EFSA, 2014).

In silico data suggest that PG has a strong binding affinity towards nuclear ERs (54 nM) (Amadasi et al., 2009). However, contradictory results have emerged from *in vitro* studies regarding its ability to induce transactivation upon ER binding. PG displayed either pure antagonistic (Amadasi et al., 2009), agonistic (ter Veld et al., 2006) or agonistic–antagonistic (Pop et al., 2014) activity in luciferase reporter *in vitro* systems. These data, correlated with the conclusion of other studies (Fang et al., 2003; Tamura et al., 2006), according to which ER ligands could also bind to androgen receptors (AR), suggest the possibility

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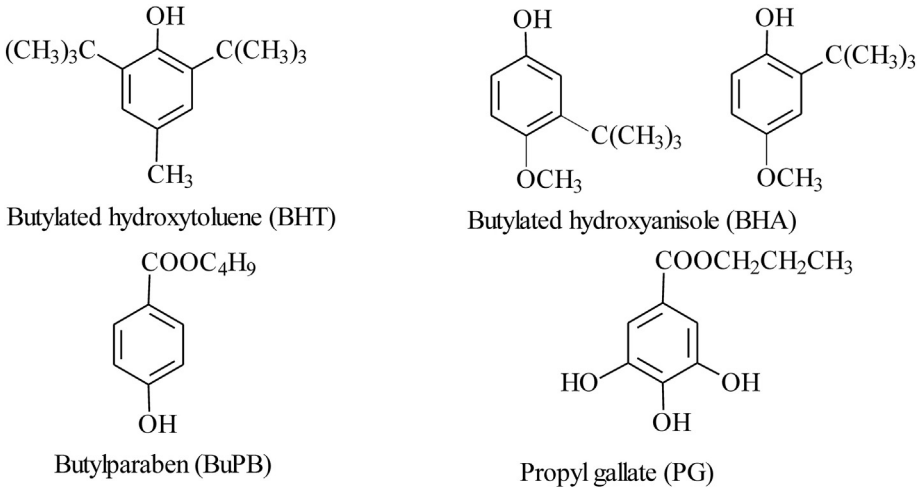

Fig. 1. Chemical structure of the selected compounds.

that PG may act as a potential ligand for the ARs. However, to date, no studies (*in vitro* or *in vivo*) have been published regarding the (anti)androgenic potential of PG.

BHA and BHT are antioxidants present in food, food packaging materials and cosmetics. *In vitro*, these compounds displayed weak androgen agonist activity (Jeong et al., 2006; Mertl et al., 2014) or partial androgen antagonist activity (Schrader and Cooke, 2000). A rat one-generation reproductive and developmental toxicity study suggested that BHA can act as an anti-androgenic compound (Jeong et al., 2005), However, BHA had no *in vivo* effects on androgen-dependent accessory sex organ weights or on testosterone levels in the Hershberger assay (Hwan et al., 2005). To our best knowledge, there are no published *in vivo* data evaluating the (anti)androgenic effects of BHT. Furthermore, no published data regarding the concentrations of BHA, BHT and PG in human biological fluids after exposure from food are available.

Parabens are widely used as antimicrobial preservatives in personal care products, pharmaceuticals and as food additives (Masten, 2005). Estimations of human exposure to parabens were reported in the literature, but the values vary considerably (from 1.26 mg/kg/day to over 140 mg/kg/day) (Błędzka et al., 2014). It is estimated that in case of dermal exposure to butyl paraben (BuPB) (Fig. 1), up to 2% of the parent compound can reach the systemic circulation (Boberg et al., 2010). Regarding the (anti)androgenic potential of BuPB, the data are inconsistent and the compound was classified as an anti-androgen (Chen et al., 2007; Satoh et al., 2005), but these results were not confirmed by further studies (Kjærstad et al., 2010).

A very important issue related to EDs is the fact that humans are simultaneously exposed to multiple chemicals. Therefore, a more realistic estimation of the potential impact of these chemicals on humans and wildlife requires the evaluation of the endocrine disruptive potential of mixtures (Kjærstad et al., 2010; Kortenkamp, 2007; Orton et al., 2014). Due to the large number of possible mixtures to which humans are exposed, experimental mixture testing is practically not possible due to the high number of potential permutations and a less time and cost-consuming approach is to develop mathematical models in order to predict the effect of mixtures (Hadrup et al., 2013; Rider et al., 2008; Thorpe et al., 2006).

The aim of the present study was to evaluate the *in vitro* (anti)androgenic activity of the selected food additives and cosmetic preservatives for single compounds and for binary mixtures. We focused our research on PG, BHA, BHT and BuPB, since their presence in products used/consumed on a daily basis might be associated with a considerable risk of human exposure. Another objective was to evaluate the possibility to estimate the mixtures' effect by applying two mathematical models (dose addition (DA) or response addition (RA)) (Rider et al., 2008). The need for individual exposure tests derived partially from the lack of data (in case of PG), but also because this information represented the starting point for estimating the effect of mixtures through mathematical modeling.

2. Materials and methods

2.1. Reagents

The test compounds (BHA, BHT, PG and BuPB), 5-alpha-dihydrotestosterone (DHT), resazurin, tricine, EDTA, DTT, ATP, Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12) were all purchased from Sigma Aldrich (Steinheim, Germany). 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA) and Tris were obtained from Fluka (Buchs, Switzerland) and luciferin (≥99%) was purchased from Roth (Karlsruhe, Germany). $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$ was obtained from Acros Organics (Geel, Belgium). L-15 culture medium was purchased from ATCC (USA). Dulbecco's Phosphate Buffered Saline (PBS) was purchased from Invitrogen, while trypsin, Leibovitz's phenol-red free medium and charcoal stripped fetal bovine serum (FBS) were all purchased from Gibco (Paisley, UK). All solvents and reagents used were of analytical grade.

2.2. Cell culture

MDA-kb2 human breast cancer cells (ATCC CRL-2713, human breast cancer cell line positive for androgen and glucocorticoid receptors) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown at 37 °C under humidified atmosphere, without additional CO_2, in L-15 medium supplemented with 10% FBS and were passaged every 2 to 3 days. Dulbecco's Phosphate Buffered Saline was used in the washing steps, while trypsin was used to detach cells from culture flasks. The potential for phenol red to interfere with the AR mediated response was suggested.(Ermler et al., 2010), therefore in the present study, during the (anti)androgenicity assay (the exposure phase to the selected compounds), the cells were plated in phenol red-free Leibovitz's medium containing 10% charcoal-stripped FBS and allowed to attach 24 h before exposure to test chemicals.

2.3. Preparation of test compounds and binary mixtures

All compounds were prepared as 1000 μL stock solutions in dimethyl sulfoxide (DMSO, ≥99.5%, Riedel-de Haën, Seelze, Germany) at a concentration of 500 mM. Working solutions of 0, 0.15, 0.5, 1.5, 5, 15, 30,